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Ozone tolerance and antioxidant enzyme activity in soybean cultivars

Tatyana Chernikova[1], J. Michael Robinson[2], Edward H. Lee[3] & Charles L. Mulchi[1,*]
[1]*Department of Natural Resource Sciences and Landscape Architecture, University of Maryland, College Park, MD 20742, USA;* [2]*USDA-ARS, Climate Stress Laboratory, Beltsville, MD 20705, USA;* [3]*Institute of Life Sciences, National Central University, Chung-Li, Taiwan 32054;* [*]*Author for correspondence*

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Abstract

The current study confirmed earlier conclusions regarding differential ozone (O_3) tolerances of two soybean cultivars, Essex and Forrest, and evaluated antioxidant enzyme activities of these two varieties based on their performance under environmentally relevant, elevated O_3 conditions. The experiment was conducted in open-top chambers in the field during the 1994 and 1995 growing seasons. Exposure of plants to moderately high O_3 levels (62.9 nl l^{-1} air, 2-year seasonal average) caused chlorophyll loss and increased membrane permeability when compared to control plants grown in charcoal filtered air (24.2 nl l^{-1} air). The other effects of O_3 treatment were decrease in seed yield, loss of total sulfhydryl groups, reduction of soluble protein content, and increase in guaiacol peroxidase activity in leaves of both cultivars. The O_3-induced increase in guaiacol peroxidase activity was much smaller in cv. Essex leaflets. Cv. Essex had less leaf oxidative damage and smaller reduction in seed yield than cv. Forrest under elevated O_3 conditions. During ozonation, mature leaflets of the more O_3 tolerant cv. Essex had higher levels of glutathione reductase (30%), ascorbate peroxidase (13%), and superoxide dismutase (45%) activity than did mature leaflets of cv. Forrest. Cu,Zn-superoxide dismutase, which represented 95% of total superoxide dismutase activity in the two cultivars, appeared to be increased by O_3 exposure in the leaflets of O_3 tolerant cv. Essex but not in those of cv. Forrest. Cytosolic ascorbate peroxidase activity was also higher in leaflets of cv. Essex than in cv. Forrest regardless of O_3 level. Stromal ascorbate peroxidase and Mn-superoxide dismutase activity did not appear to be involved in the O_3 tolerance of the two soybean cultivars.

Abbreviations: APX (cAPX/sAPX) – ascorbate peroxidase (cytosolic/stromal isoform); AsA – ascorbate; CF – charcoal filtered air; Chl – chlorophyll; p-CMS – para-chloromercurisulfonic acid; DHAR – dehydroascorbate reductase; DTNB – 5,5′-dithiobis (2-nitrobenzoate); GPX – guaiacol peroxidase; GR – glutathione reductase; GSSG – oxidized glutathione; H_2O_2 – hydrogen peroxide; MDHAR – monodehydroascorbate reductase; NBT – nitroblue tetrazolium; NF – non-filtered air; O_2^- – superoxide ion; O_3 – ozone; $OH^{\cdot}/OH^-$ – hydroxyl radical/ion; OTC – open-top chamber; *p* – level of statistical probability; SDS – sodium dodecyl sulfate; -SH – sulfhydryl (thiol) group; SOD (Cu,Zn-SOD/Fe-SOD/Mn-SOD) – superoxide dismutase (Cu,Zn isoform/Fe isoform/Mn soform); SPAD unit – ratio of optical densities at 650 and 940 nm after light transmission

Introduction

There is considerable evidence for the presence of an ascorbate-glutathione cycle in chloroplasts (Foyer and Halliwell 1976; Asada 1984; Dalton 1991; Foyer et al. 1991). The cycle is responsible for scavenging toxic H_2O_2 produced during the photochemical reduction of O_2 in thylakoids (Mehler 1951). The immediate product of O_2 reduction is O_2^- that dismutates into H_2O_2 in the stroma as a result of SOD catalysis (Foyer et al. 1991). The first stage of the ascorbate-glutathione cycle is mediated by APX, the enzyme responsible for catalyzing the reduction of H_2O_2 by AsA. Dehydroascorbate and monodehydroascorbate

produced in the APX reaction are both recycled to AsA by a consortium of enzymes including DHAR, MDHAR, and GR (Dalton 1991; Foyer et al. 1991). Glutathione reductase and MDHAR employ NADPH as a reductant that is derived from the light-dependent reactions in the chloroplast (Dalton 1991; Foyer et al. 1991), or from the oxidative pentose phosphate pathway (Dizengremel and Pétrini 1994). Jablonski and Anderson (1981) suggested that GR is a rate-limiting enzyme in the ascorbate-glutathione cycle.

Recent reports indicate that the cycle components are also present in the cytosol of plant cells (Edwards et al. 1990; Asada 1992) where they detoxify H_2O_2 leaked from various cellular compartments. Recent studies of plant defense functions against environmental stresses have been found to involve cytosolic and chloroplastic isoforms of antioxidant enzymes. For example, it was found that oxidative stress damage was significantly reduced in transgenic plants with elevated levels of expression for chloroplastic GR (Aono et al. 1993; Foyer at el. 1995) and SOD genes (Gupta et al. 1993; Van Camp et al. 1994).

In contrast to the majority of internal oxidative stresses that are initiated in chloroplast, O_3 pollution is considered to be an external stress. Ozone uptake occurs through the leaf stomata. However, O_3 concentrations inside the cells appear to be very low (Laisk et al. 1989) suggesting that most of the O_3 which has entered intercellular spaces is broken down in the cell wall and plasmalemma while toxic O_3 products, e.g. H_2O_2, are able to be transported into the cell.

Symptoms of O_3-induced damage are very similar to the symptoms of other oxidative damage with the exception that injury in O_3-exposed tissue first occurs in the apoplast and cytosol of leaf mesophyll cells. Interacting with water, O_3 degrades into O_2^-, H_2O_2, peroxyl radical, and other active O_2 species (Hoigné and Bader 1976). Presence of O_2^- and toxic $OH\cdot$ has been reported under physiological conditions following O_3 fumigation (Grimes et al. 1983; Runeckles and Vaartnou 1997). Ozone and its derivatives oxidize -SH groups of proteins and cause lipid oxidation and/or peroxidation (Hippeli and Elstner 1996). Decrease in the amounts of Rubisco and inhibition of photosynthetic activity in O_3-treated plants were reported as well (Dann and Pell 1989; Eckardt et al. 1991; Farage et al. 1991).

Differences in plant O_3 tolerance are often correlated with the differences in antioxidant enzyme activities. For example, O_3 insensitive cultivars of *Phaseolus vulgaris* had twice as much GR activity as sensitive varieties (Guri 1983). High APX activity was associated with O_3 tolerance of *Nicotiana tabacum* genotype (Batini et al. 1995). Pitcher and Zilinskas (1996) indicated that high Cu,Zn-SOD content in the leaf mesophyll cell cytosol was more important for O_3 tolerance than the enhanced activity of chloroplastic Cu,Zn-SOD isoform (Pitcher et al. 1991). Although in most cases, O_3 stimulated antioxidant enzyme activities, some studies with plants exposed to elevated O_3 have shown a decrease or no changes in the activity levels for scavenging enzymes (Bender et al. 1994).

Many experiments involving antioxidant enzyme responses to O_3 treatments were conducted in controlled growth conditions which fail to simulate daily changes in natural environment; e.g., daily cycles of natural sunlight energy. The current study was done with field-grown plants and repeated for two growing seasons. Soybean plants were chosen because limited information is available regarding antioxidant enzyme activity in soybean leaves, despite an early report indicating soybean high sensitivity to O_3 pollution (Lesser et al. 1990). Based on field studies using open-top chambers (OTCs), the two soybean cultivars (cv. Essex and cv. Forrest) used in the current study appeared to have differential O_3 tolerance. Chronic exposures of cv. Essex to elevated O_3 did not reduce seed yields, while seed yields of cv. Forrest were reduced approximately 20% in response to the long-term O_3 treatments (Mulchi et al. 1988). The first objective of the current experiment was to confirm cultivar differences in terms of O_3 response using growth and leaf injury characteristics. Secondly, it was of the interest to estimate antioxidant enzyme activities in soybean leaves and relate them to the observed differences in O_3 tolerance.

Materials and methods

Plant material and gaseous treatments

The studies were conducted on the South Farm of the Beltsville Agricultural Research Center, US Department of Agriculture, Beltsville, Maryland, USA, during 1994 and 1995. The experimental site was located on Codorus silt loam soil with a pH of 6.2.

Seeds of two soybean (*Glycine max* L.) cultivars ('Forrest' and 'Essex') were germinated in the greenhouse and then transplanted into 4 m × 4 m field plots 10 cm apart in rows spaced 0.6 m apart. The OTCs (Heagle et al. 1973) were installed and gaseous treatments were initiated soon thereafter. The air quality

treatments in the OTCs consisted of control (CF) and elevated O_3 fumigation (NF+O_3). Charcoal filters removed part of the ambient O_3 for control treatments. The high O_3 treatment consisted of O_3 artificially generated from O_2 with a Griffin O_3 Generator (Griffin Technics Corp.[1], Lodi, New Jersey) and injected into blowers where it was mixed with the ambient air prior to entering the chambers. Ozone treatments were applied 7 h day^{-1} (1000–1700 hrs EST), 5 days a week. Air samples were collected inside each chamber at the canopy level. The O_3 chamber concentrations were checked hourly with a Dasibi model 1008, UV Photometric O_3 Analyzer (see Mulchi et al. 1992, 1995 for details). Seasonal average O_3 concentrations were 23.7 and 24.6 nl l^{-1} for CF treatments and 63.4 and 62.4 nl l^{-1} for NF+O_3 treatments for 1994 and 1995, respectively.

Plants were irrigated when soil moisture levels dropped below -0.06 MPa or 14% . The average seasonal air temperature was 20.4 °C in 1994 and 21.3 °C in the 1995 growing season.

Relative Chl content

During the vegetative stage of the 1994 growing season, the middle leaflets of the uppermost trifoliates were tagged and their lengths were monitored and recorded until fully expanded. Relative Chl contents were estimated non-intrusively on the same leaflets with a SPAD-502 portable chlorophyll meter (Minolta Camera Co. Ltd, Japan) and expressed in the SPAD units. One SPAD unit is equal to the ratio of optical density at the two wavelengths, 650 nm and 940 nm, being transmitted through the leaf. Upon calibration against measured Chl values for a given species, SPAD measurements correspond to Chl levels in the leaves but should not be viewed as absolute Chl values (Yadava 1986). For soybean leaves, one SPAD unit was found to equal approximately 3.17 μg of Chl/cm^2 with a linear relationship over the range of values typically found in soybeans treated with O_3 (Lee et al. 1996).

Ion leakage

Ion leakage measurements were carried out twice during the 1994 growing season. Ten disks of 1 cm diameter were cut from two uppermost fully-expanded leaflets of each cultivar in each OTC. The disks were placed in vials containing boiling stones and mixed with 10 ml of 0.3 M mannitol. The vials were weighed, agitated on a shaker for 4 hours at 100 cycles/min and the conductivity of solutions was measured. The solutions were boiled for 5 min and left overnight at 4 °C. The following day, the weights of solutions were adjusted with distilled water to those of the corresponding solutions from the initial extraction of fresh disks. After 1 h of mixing at 100 cycles/min, the maximal conductivity of solutions was measured. Ion leakage was calculated as conductivity after 4 h of the first day ion extraction expressed as percent of maximal leaf electrolyte leakage or conductivity resulting from boiling.

Leaf sampling and crude extract preparation

Leaf disk samples for assay of antioxidant enzyme activity and protein content were collected at 47, 53, 59 and 71 day after emergence in 1994, and 65 and 87 day in 1995. Twenty disks of 2.0 cm diameter were randomly cut from four fully-expanded uppermost trifoliates of each cultivar in each OTC, frozen in liquid N_2 within 2 min of leaf detachment, and stored at -80 °C. Sampling was carried out between 1000 and 1400 h under conditions having an average temperature of 26.3 °C, relative humidity of 62%, and light levels exceeding 1100 μmol photon m^{-2} s^{-1}.

For extraction, the samples were ground to powder with liquid N_2 and mixed with 20 ml of Tris-HCl buffer, pH 7.0 containing 50 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, 1% polyvinylpyrrolidone. After 20 min of centrifugation at 10 000 g, the supernatants were filtered though a nylon membrane (0.45 μm) and used for analysis.

Samples for APX activity were extracted with the same buffer, except that they contained 1 mM AsA. The crude extracts used for APX isozyme activity measurements were partially purified through the Sephadex G-25 M prepacked disposable columns. 2.5 ml of crude extract was passed through the column equilibrated with APX extraction buffer containing 10% glycerol and washed with 3.5 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM AsA. Soluble protein content was measured before and after gel filtration.

Total -SH groups and soluble protein

Total -SH groups in soybean leaflet samples were measured employing the method described by DeKok

[1] Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

et al. (1985). The plant extract (0.5 ml) prepared as described above was treated with 1.9% SDS in 95 mM Tris buffer, pH 8.0 to denature the extract proteins. Following additions of 0.1 ml of 10 mM DTNB in 20 mM potassium phosphate buffer (pH 7.0) and a 15 min incubation at room temperature, absorbance was measured spectrophotometrically at 412 nm. The readings were corrected for DTNB absorbance in the absence of extract. Total -SH group concentrations were calculated using the mM extinction coefficient 13.6 mM^{-1} cm^{-1} (Peters et al. 1988).

Soluble protein concentrations were measured using the Bradford protein microassay with BSA as a standard (Bradford 1976).

Enzyme assays

Activities of GPX, APX, SOD and GR were measured with a Shimadzu Model UV160U spectrophotometer (Shimadzu, Japan). The concentrations of reaction mixtures components, e.g. substrates for GPX, APX, SOD, and GR were experimentally optimized to support maximum catalytic activity for each of those enzymes.

Guaiacol peroxidase activity was measured as described by Maehly and Chance (1959) in 3 ml of reaction mixtures containing 3.5 mM phosphate buffer (pH 7.0), 6.7 mM guaiacol and 50 μl of leaf extract at 25 °C. The reaction was started by the addition of 20 μl of 22.5 mM H_2O_2. The increase of absorbance at 470 nm was measured and the activity was calculated as μmol of tetraguaiacol production per second using the mM extinction coefficient for tetraguaiacol of 26.6 mM^{-1} cm^{-1}. The mixture without enzyme extract was used as a control. Incubation of reaction mixture with 50 μM p-CMS revealed no guaiacol consumption by APX.

Total APX activity was measured as described by Asada (1984) with modifications according to the method described by Amako et al. (1994). The reaction mixture (1 ml) contained 50 mM phosphate buffer, pH 7.0, 0.5 mM AsA and 50 μl of leaf extract at 25 °C. Ascorbate peroxidase activity was measured at 290 nm for 1 min after addition of 20 μl 10 mM H_2O_2. Nonspecific APX activity was measured after 10 min of incubation of reaction mixture with 50 μM p-CMS. The mixture without enzyme extract served as a control. The activity was calculated as μmol of AsA consumption per second minus nonspecific APX activity using the mM extinction coefficient 2.8 mM^{-1} cm^{-1}.

Cytosolic and stromal APX activity was measured by the procedure described by Amako et al. (1994) where 50 μl of leaf extract was added to 5 ml of N_2-aerated 50 mM potassium phosphate buffer, pH 7.0, containing 10 μM H_2O_2. In 3, 4, 5, and 6 min intervals, samples were withdrawn and 5 μl of 100 mM AsA was added. The residual APX activities were assayed after addition of 10 μl 10 mM H_2O_2. Measured APX activity was plotted vs. time of anaerobic H_2O_2 inactivation of APX and interpolated to time zero. Stromal APX activity was equal to the difference between maximal APX activity (measured without p-CMS) and the APX activity at time zero. Cytosolic APX activity was calculated as a difference between activity at time zero and APX activity in presence of p-CMS (non-specific APX activity).

Glutathione reductase activity was measured at 30 °C in the reaction mixture (1 ml) containing 0.1 M Hepes buffer, pH 7.6, 1 mM EDTA, 0.1 mM NADPH, 1 mM GSSG and 25 μl of leaf extract (Klapheck et al. 1990). The reaction was started with the addition of NADPH, and the decrease of absorbance at 340 nm due to oxidation of NADPH was monitored. The rate calculations employed the mM extinction coefficient for NADPH of 6.2 mM^{-1} cm^{-1}. A control without GSSG was used to correct for the NADPH oxidase reaction which, in this study, appeared to be negligible.

The activity of SOD was assayed by measuring the SOD inhibition of the photochemical reduction of NBT (Beauchamp and Fridovich 1971). Each 3 ml reaction mixture contained 50 mM phosphate buffer, pH 7.8, 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and 20 μl of leaf extract. Riboflavin was added last after the samples were incubated for 10 min under a 15 W fluorescent lamp to reach a constant temperature of 30 °C. The reaction was run for 15 min and stopped by switching the light off. The absorbance by the reaction mixture at 560 nm was read. A mixture without the extract was used as a control and a dark control mixture served as blank. The activity was calculated as described by Giannopolitis and Ries (1977) as SOD Units/ml = $(V/v - 1)$(dilution factor), where V is the rate of reaction without enzyme and v is the rate of reaction in the presence of enzyme. The linearity of the time effect and amount of protein extract were tested prior to the analysis. Since no Fe-SOD was detected in the soybean leaf extract (see below), Mn-SOD activity was measured with the method described above after 10 min incubation of plant extract with 2 mM

KCN. Copper,Zn-SOD activity was calculated as a difference between total SOD and Mn-SOD activities.

Native PAGE and SOD activity staining

Polyacrylamide gels were prepared as described by Laemmli (1970) except that SDS was omitted. Separating gels had 7.5% final acrylamide. Equal amounts of protein for each treatment were loaded and subjected to separation under non-denaturing conditions at 4 °C for 4 h at the constant current 25 mA per gel.

Staining for SOD activity was carried out as described by Beauchamp and Fridovich (1971). The gels were incubated in 2.45 mM NBT for 20 min and 15 min in a solution containing 50 mM potassium phosphate buffer, pH 7.8, 0.028 M TEMED (N,N,N′,N′-tetramethylethylenediamine) and 0.028 mM riboflavin. Superoxide dismutase was localized after illumination of gels by fluorescent light. The gels were photographed and vacuum dried. Pre-incubation of gels with 2 mM KCN allowed the detection of Mn and Fe-SODs. Pretreatment of gels with 0.015% H_2O_2 revealed Mn-SOD isozymes only.

Statistical methods

The experiment described above was conducted as a randomized complete block design with two field replicates in 1994 and three replicates in the 1995 growing season. Ozone treatments were randomly assigned to the OTCs, and cultivars were planted in a split-plot fashion within the OTCs. Data were analyzed statistically using Proc Mixed of the SAS package (SAS Institute Inc., Cary, North Carolina). Significance of main effects (O_3, cv) and their interaction ($O_3 \times$cv) were reported at $P \leq 0.05$. Mean separation was conducted using pairwise comparisons at $P \leq 0.05$. Ozone effects on biochemical damage characteristics were compared with zero using a *t*-test.

Relative chlorophyll concentration data of each plant were fitted to the second degree polynomials for centered data using regression techniques in the SAS package. The regression parameters of generated curves of different treatments were compared using Proc Mixed.

Results

Relative Chl content and seed yield

In the current study, measurements with the SPAD meter were employed to estimate relative Chl content in relationship to O_3 leaf injury over the period of leaf development (for the definition of the SPAD unit see Materials and methods). Young leaves (10–14 days old) of both cultivars did not show any symptoms of leaf injury or Chl loss due to O_3 exposure (Figure 1). Measurable differences in relative Chl content between O_3-treated and control plants were detectable by the 15th day of observation which coincided with the time when the soybean leaflets reached maximal leaf area. Between 20 and 35 days, SPAD monitoring indicated that Chl levels in leaflets of O_3-treated cv. Forrest declined more rapidly that in leaflets of cv. Essex (Figure 1). During the same period, there was no difference between the cultivars in decline of Chl content in leaflets of the control plants. On the 20th day, leaflets of O_3-treated cv. Forrest had about 20% less SPAD units than O_3-treated cv. Essex. On the 35th day, the SPAD units had declined to 18.1 and 36.6 for the O_3-treated cv. Forrest and cv. Essex, respectively (Figure 1).

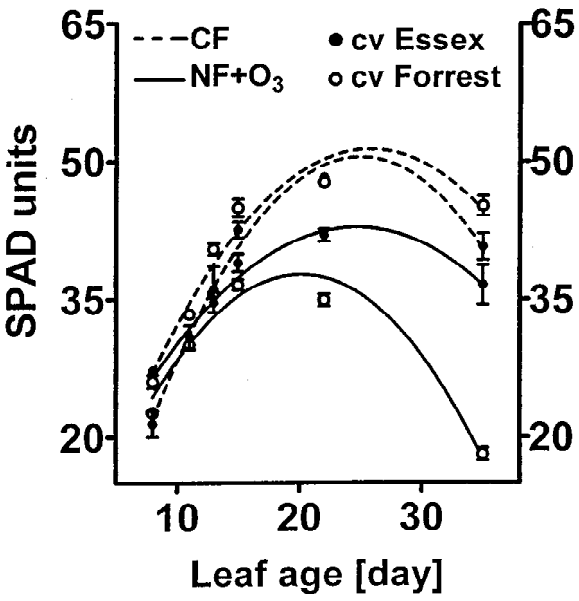

Figure 1. Relative Chl concentration expressed in SPAD units (one SPAD unit $\approx$ 3.17 μg of Chl/cm^2) as a function of leaf age for two soybean cultivars (n = 24). Leaf age was defined arbitrary starting the day when leaf became visible. Data were fitted to second degree polynomials: $Y = a + bX + cX^2$.

The seed yield data in the current study were collected to compare the influence of O_3 on plant growth in both cultivars. Analysis of variance indicated that seed yield was significantly affected by O_3 treatment (P = 0.019 for O_3 effect in F-test). As shown in Fig-

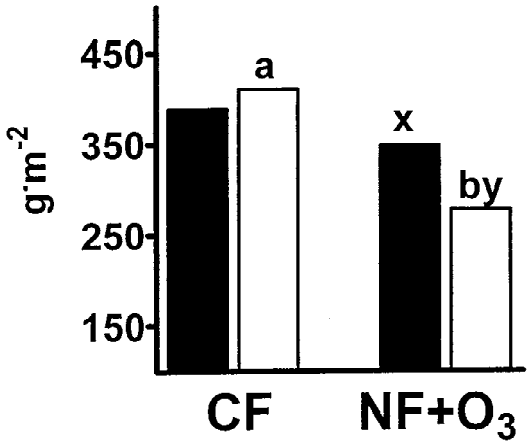

Figure 2. Seed yield (from 20 plants) of two soybean cultivars (■ – cv. Essex and □ – cv. Forrest) averaged over two growing season. The letters *a* and *b* indicate significant ($P \leq 0.01$) differences between treatment means within each cultivar. The letters *x* and *y* indicate significant ($P \leq 0.1$) differences between cultivar means within O_3 treatment or control. Means without letters are not significantly ($P > 0.1$) different.

Table 1. Comparison of the O_3-induced changes in the mature leaves of two soybean cultivars. Influence of O_3 on (A) leaflet protein levels, (B) leaflet sulfhydryl levels, and (C) ion leakage from leaflets

Cultivar	Treatments	
	CF	NF+O_3
A. Leaflet protein levels (mg soluble protein m^{-2})		
cv. Essex	502[a]	449[b]
cv. Forrest	543[a]	434[b]
B. Leaflet total -SH groups (mmol -SH m^{-2})		
cv. Essex	19.3	13.8
cv. Forrest	20.0[a]	11.8[b]
C. Ion leakage from leaflets (% of maximal conductance[1])		
cv. Essex	15.1	16.4
cv. Forrest	14.1[a+]	17.3[b+]

[1]Maximum conductance was equivalent to 120–390 mhos ml^{-1}.
The letters a and b indicate significant ($P \leq 0.05$, $+P \leq 0.1$) differences between treatment means within each cultivar.

ure 2, the magnitude of the O_3-induced seed yield reduction was different in two cultivars. Control plants of cv. Forrest and cv. Essex appeared to have similar seed yield. In contrast, the seed yield of O_3-treated cv. Forrest was 32% less, while the seed yield of O_3-treated cv. Essex was only 10% less than the seed yield of the control plants (Figure 2).

Ozone internal damage

Mature leaflets located on the top of the canopy late in the season (reproductive period) were tested for soluble protein and total -SH group content. Ozone exposure significantly ($P = 0.01$) reduced soluble protein concentration in the soybean leaves of both cultivars (Table 1A), but cv. Forrest was more severely affected than cv. Essex. Leaflet soluble protein level in O_3-treated cv. Forrest was 20% lower than protein levels in control leaflets. In leaflets of O_3-treated cv. Essex, soluble protein content was 11% less than the controls (Table 1A).

Compared with control plants, O_3 exposure decreased total -SH content in soybean leaflets of by 41% for cv. Forrest (Table 1B). In the leaflets of cv. Essex this decrease in sulfhydryl group was not statistically significant even at 0.1 probability level.

Membrane injury in the soybean cultivar leaflets caused by O_3 exposure was estimated by the electrolyte leakage data (see methods). Ion leakage from leaves of control and O_3-treated cv. Essex were similar (Table 1C). However, O_3 treatment of cv. Forrest plants resulted in 23% increase in ion leakage compared with that of the control leaves (Table 1C).

Total activities of GPX, SOD, APX, and GR

Expression of total enzyme activities per m^2 of the leaf area was chosen in the current study to produce results comparable to the gas-exchange (photosynthesis, stomatal conductance, O_3 uptake) numbers frequently published in various reports. Such results were similar to the results obtained through the more conventional way of total enzyme activity expression – on fresh weight basis (data not shown).

Activity of GPX was highly induced by O_3 treatment in the leaves of both cultivars during the 1994 and 1995 vegetation seasons. As shown in Table 2, the two cultivars had different levels of GPX activity resulting from O_3 treatment. For cv. Forrest, such increase was higher (3.85 μmol s^{-1} m^{-2}) than for cv. Essex (2.57 μmol s^{-1} m^{-2}). Peroxidase activity measurements using spectroscopic analysis and native gel staining have shown that the differences in the O_3-induced GPX activity increases between cultivars was greater for older leaves late in the season (data not shown).

Figure 3 summarizes total SOD, APX, and GR enzyme activities in soybean leaves averaged over two vegetative seasons. Analysis of variances employed in

Table 2. Comparison of the O_3-induced changes in the mature leaves of two soybean cultivars. Influence of O_3 on leaflet guaiacol peroxidase

Cultivar	Treatments	
	CF	NF+O_3
	μmol s^{-1} m^{-2}	
cv. Essex	6.55^{b}	9.12^{ax}
cv. Forrest	6.13^{b}	9.98^{ay}

The letters a and b indicate significant ($P \leq 0.05$) differences between treatment means within each cultivar.
The letters x and y indicate significant ($P \leq 0.05$) differences between cultivar means within O_3 treatment or control.

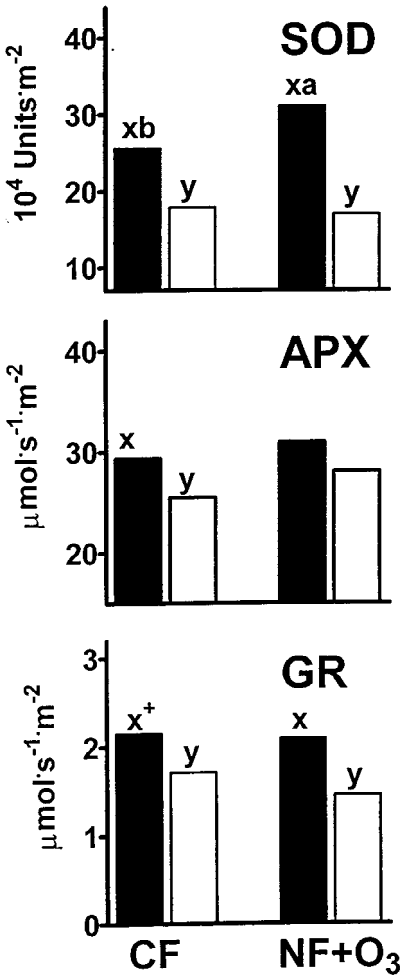

Figure 3. Total SOD, APX, and GR activities in the leaves of two soybean cultivars (■ – cv. Essex and □ – cv. Forrest, $n = 78$). The letters *a* and *b* indicate significant ($P \leq 0.1$) differences between treatment means within each cultivar. The letters *x* and *y* indicate significant ($P \leq 0.05$, $+P \leq 0.1$) differences between cultivar means within O_3 treatment or control. Means without letters are not significantly ($P > 0.1$) different.

the data analysis revealed that the O_3 treatment did not have a significant effect on antioxidant enzyme activities in the leaves of the two cultivars. However, the two cultivars had different antioxidant enzyme activities at both O_3 levels.

In the current study, SOD activity in soybean leaves varied between 17×10^4 to 31×10^4 Units m^{-2} (see methods for Unit definition). Significant ($P \leq 0.001$) differences in total SOD activities were reported for the two cultivars. The leaves of cv. Essex control plants had 30% more SOD activity than cv. Forrest control plants. The differences became more apparent under high O_3 conditions (Figure 3). Total SOD activity in cv. Essex slightly increased due to O_3 exposure while SOD activity in cv. Forrest remained unchanged. This resulted in a 45% difference between SOD activity levels of the two cultivars under high O_3 treatment.

Average seasonal APX activity in soybean leaves was around 28 μmol s^{-1} m^{-2}. There was a trend in O_3 stimulation of APX activity in the two cultivars (Figure 3). The differences in APX activity levels for the two cultivars were less apparent than in case of SOD but were still significant (F-test for cv. effect $P = 0.05$) when results were averaged over two O_3 levels. Control plants of cv. Essex had 13% more total APX activity than the leaves of cv. Forrest (Figure 3).

Glutathione reductase activity in soybean leaves varied around 1.8 μmol s^{-1} m^{-2} and was little affected by the experimental O_3 concentrations. However, when GR activities were averaged over all O_3 treatments cv. Essex had 26% more GR activity than cv. Forrest. Thus, under control conditions, cv. Essex had 2.15 μmol s^{-1} m^{-2} of GR activity as opposed to 1.71 μmol s^{-1} m^{-2} of GR activity in cv. Forrest. As result of O_3 exposure, GR activity in cv. Forrest tended to decrease to 1.45 μmol s^{-1} m^{-2} which was 30% lower than GR activity recorded for O_3-treated leaves of cv. Essex (Figure 3).

SOD isozyme activities

Superoxide dismutase activity staining of native protein gels was done to determine what SOD isoforms were present in soybean leaves. Examination of SOD

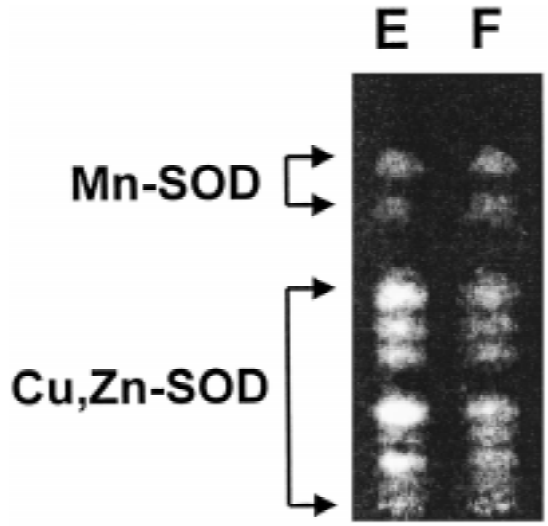

Figure 4. Native gel stained for SOD activity in the mature leaves of two soybean cultivars (E – Cv. Essex and F – Cv. Forrest) growing under ambient O_3 level. The gel was run in 7.5% polyacrylamide. Each well contained 50 μg protein.

Table 3. Superoxide dismutase activities of (A) Cu,Zn and (B) Mn-containing isozymes in the mature leaves of two soybean cultivars in response to O_3 treatment

Cultivar	Treatments	
	CF	NF+O_3
	10^4 Units m^{-2}	
A. Cu,Zn-SOD		
cv. Essex	27.2[b]	39.8[ax]
cv. Forrest	23.7	21.7[y]
B. Mn-SOD		
cv. Essex	2.1	1.3
cv. Forrest	1.9[a]	0.5[b]

The letters a and b indicate significant ($P \leq 0.1$) differences between treatment means within each cultivar.
The letters x and y indicate significant ($P \leq 0.05$) differences between cultivar means within O_3 treatment or control.

zymograms revealed ten SOD bands with eight of them being CN^- and H_2O_2 sensitive which corresponded to Cu,Zn-SODs (Figure 4). At least two Mn-SOD isozymes (CN^- and H_2O_2 insensitive) and no Fe-SODs (CN^- sensitive, H_2O_2 insensitive) were detected on the SOD gel. The lower Mn-SOD band was less defined in cv. Essex than in cv. Forrest. However, overall Cu,Zn-SOD activity appeared to be greater in cv. Essex than in cv. Forrest (Figure 4).

Quantitative measurements of Cu,Zn- and Mn-SOD activities were performed on the samples collected in the 1995 growing season (Table 3). Absence of Fe-SOD allowed separation and quantification of Cu,Zn and Mn containing SOD activities under standard assay conditions (Beauchamp and Fridovich 1971) using CN^- for activity differentiation. The Cu,Zn-SOD isozymes constituted approximately 95% of the total activity. The changes in SOD activity shown in Figure 3 appear to be consistent with the changes in Cu,Zn-SOD activity presented in Table 3.

Copper,Zn-SOD activity increased from 27.2×10^4 to 39.8×10^4 Units m^{-2} in cv. Essex but tended to decrease in the leaves of cv. Forrest upon increasing the levels of O_3 exposure (Table 3). Control plants of cv. Essex had slightly more Cu,Zn-SOD activity than plants of cv. Forrest. However, under high O_3 exposures cv. Essex had 46% more Cu,Zn-SOD activity than cv. Forrest. There were no significant differences between Mn-SOD activities in the leaves of two cultivars. The results shown in Table 3 suggested that O_3 tended to reduce Mn-SOD activity especially in cv. Forrest.

APX isozyme activities

Application of the method by Amako et al. (1994) in the current study allowed separation and quantification of two major APX activities in leaves – chloroplastic stromal and cytosolic APXs – using a commonly used APX spectroscopic assay (Asada 1982). As a result of APX isozyme activity measurements, an average cAPX/sAPX ratio in the soybean leaves was recorded as 2.8. Changes in total APX activity shown in Figure 3 appear to be consistent with the changes in cAPX activity listed in Table 4. Exposure to elevated O_3 tended to increase cAPX activity in the two cultivars by an average 24%. Under all O_3 treatments, cv. Essex maintained higher cAPX activity than cv. Forrest (F-test for cv. $P = 0.019$). Thus, control cv. Essex plants had 26% more cAPX activity and ozonated cv. Essex plants had 15% more cAPX activity than the plants of cv. Forrest. Stromal APX activities tended to be inhibited by elevated O_3 exposures (Table 4). However, high variability of the sAPX activity results did not allow us to determine O_3 and cultivar effect on the activity of this isozyme.

Table 4. Ascorbate peroxidase activity of (A) cytosolic and (B) stromal isozymes in the mature leaves of two soybean cultivars in response to O_3 treatment

Cultivar	Treatments	
	CF	NF+O_3
	μmol s^{-1} m^{-2}	
A. cAPX		
cv. Essex	25.7^{ax}	29.9^{bx}
cv. Forrest	19.0^{y}	25.3^{y}
B. sAPX		
cv. Essex	12.25	4.75
cv. Forrest	11.25	7.33

The letters a and b indicate significant ($P \leq 0.05$) differences between treatment means within each cultivar.
The letters x and y indicate significant ($P \leq 0.05$) differences between cultivar means within O_3 treatment or control.

Discussion

Deleterious effects for O_3 on plant cells are closely related to an oxidative nature of O_3 molecules. Symptoms of O_3 damage commonly observed on the leaves are primarily a result of Chl destruction and necrotic cell death. Loss of Chl content in the O_3-treated leaves was reported earlier in *Triticum aestivum* (Nie et al. 1993) and *Phaseolus vulgaris* (Guzy and Heath 1993). In the current study, O_3 decreased SPAD estimates of total Chl in the soybean leaves may be directly correlated with reductions in the leaf Chl content (Yadava 1986). Ozone-induced Chl breakdown observed under the current experimental conditions might be symptomatic of premature senescence and could be associated with oxidative conditions within the cells.

Seed yield loss is also one of the potential impacts of the plant stress. Decreases in seed yield under high O_3 exposures have been reported for variety of plants (Lesser et al. 1990). Growth reductions in response to O_3 stress are often a result of photosynthesis inhibition (Farage et al. 1991) and/or increased respiratory activity for repair and maintenance (Amthor and Cumming 1987). Decreases in Rubisco content appeared to be one of the reasons for lowered photosynthetic capacity of O_3-treated plants (Dann and Pell 1989). Eckardt et al. (1991) reported that losses in the Rubisco content and its carboxylation activity were correlated with the decline in the -SH concentrations of Rubisco protein. There is still limited information available regarding changes in the total leaf -SH content under O_3 conditions. The -SH loss observed in the current study in O_3-treated plants may be a result of direct and indirect interactions of O_3 with -SH groups of proteins and other non-protein sulfhydryl compounds (Hippeli and Elstner 1996). Reduction in soluble protein content will be a consequence of oxidative modifications of proteins and their proteolytic destruction. In older leaves, decreases in soluble protein concentrations are related to accelerated senescence processes by O_3.

The ability of O_3 and/or its products to interact with lipid components may have a destructive effect on cell membranes. Heath (1987) reported an increase in membrane permeability in *Chlorella sorokiniana* exposed to low O_3 levels. Such membrane perturbations may be caused not only by lipid structural alterations but also by modifications of membrane proteins. Dominy and Heath (1985) reported that O_3 inhibition of K^+-ATPase of plasmalemma was correlated with oxidation of protein -SH groups and formation of disulfide bridges. Changes in membrane permeability in the current study were estimated by measurements of ion leakage from leaf tissue in mannitol solution. The effect of O_3 treatments on membrane integrity appeared to be significant only for cv. Forrest suggesting that a degree of O_3-induced membrane damage differed in the two cultivars. In addition to the ion efflux data, cv. Forrest had greater O_3 internal injury than cv. Essex as characterized by decrease in -SH and soluble protein content in mature O_3-exposed leaves.

Differences in O_3 sensitivities of the two soybean cultivars may be confirmed by GPX results. The induction in GPX activity was often reported in response to external stresses and associated with hypersensitive cellular response (Campa 1991) and tissue wounding (Kawaoka et al. 1994). Tingey et al. (1976) reported faster induction of GPX and other lesion formation enzymes in an O_3 sensitive soybean cultivar than in an O_3 tolerant one. In the current study, GPX activity can be used to evaluate an overall injury level of soybean leaves. Peroxidase activity results in Table 2 suggested that overall stress injury in the leaves of cv. Forrest was greater than in cv. Essex. It is logical that greater levels of internal damage to cv. Forrest than cv. Essex under high O_3 conditions would require larger amounts of energy inputs for maintenance and repair which would ultimately reduce amounts of carbohydrates available for seed production. Seed yield results along with relative Chl data confirmed the differences in two soybean cultivars in terms of

O_3 sensitivities with cv. Essex being more O_3 tolerant than cv. Forrest.

Statistical analysis used in the study to evaluate antioxidant enzyme activities suggests distinct differences exist between the two soybean cultivars. Figure 3 clearly illustrates some of them. Average levels of total SOD, APX, and GR activities appeared to be higher in cv. Essex than in cv. Forrest. Under high O_3 conditions, cv. Essex had higher SOD and GR activities than cv. Forrest. Differences in the antioxidant enzyme activities of these two cultivars may determine cultivar differential responses to the O_3 treatments which traditionally have been estimated by internal damage and seed yield losses. Some of the early works related total antioxidant activity levels to O_3 tolerance. Thus, O_3 tolerant cultivars of *Phaseolus vulgaris* and *Nicotiana tabacum* appeared to have higher and more stable levels of GR activity than sensitive varieties (Guri 1983; Tanaka et al. 1990). Batini et al. (1995) determined a positive correlation between the EDU-induced overall APX activity and O_3 tolerance in *Nicotiana tabacum.* They also reported that an O_3 tolerant cultivar had more APX activity than an O_3 sensitive type. However, there was no correlation between total SOD activity and O_3 tolerance in *Glycine max* (Sheng et al. 1993) and *Nicotiana tabacum* (Tanaka et al. 1990). Malan et al. (1990) reported that in order to be tolerant to oxidative stress, plants must exhibit elevated activities of both GR and SOD. High activities of only GR or SOD were not sufficient to reduce stress damage. Apparently, such observations may be partially explained by the toxic nature of the primary product for SOD enzymatic reactions, H_2O_2, and by the necessity for continuous AsA regeneration.

Activity staining of polyacrylamide gels revealed two SOD groups, Cu,Zn-SOD and Mn-SOD, in the multiple isozymes. Iron SOD is the SOD isozyme (prokaryotic type) located mostly in chloroplast (Scandalios 1994). Corpas et al. (1991) did not find any Fe-SOD activity in several leguminous species and no Fe-SOD was detected in soybean leaves in the current study. Copper,Zn-SOD is usually located in cytosol and chloroplast of green plants (Scandalios 1994). The current results indicated that a majority of the SOD was associated with Cu,Zn-SOD. Enhanced activity of Cu,Zn-SOD in cv. Essex observed under current experimental conditions may improve overall capacity of the cultivar to scavenge toxic O_2^- when compared with the more O_3 sensitive cv. Forrest. The study did not determine a relative significance of cytosolic vs. chloroplastic Cu,Zn-SODs in the two cultivars regarding O_3 tolerance. However, earlier reports have indicated that cytosolic Cu,Zn-SOD is the form most responsive to oxidative stresses (Matters and Scandalios 1987; Pitcher and Zilinskas 1996) and appeared to be more important for O_3 tolerance than the activity of the chloroplastic isoform (Pitcher et al. 1991; Pitcher and Zilinskas 1996).

Manganese-SOD is considered to be mostly a mitochondrial isoform (Scandalios 1994). In mature soybean leaves during the reproductive stages of plant development, Mn-SOD and sAPX tended to be inhibited by the long-term O_3 exposure. Such trends need to be confirmed to understand whether O_3 negative effects on intercellular processes (mitochondrial respiration and electron transport in the chloroplast) are related to the inhibitions of compartmentalized antioxidant enzyme activities and level of their expression (Conklin and Last 1995). Low relative activity of the Mn-SOD isoform and no activity induction in response to the O_3 treatments suggest an insignificant role for Mn-SOD in O_3 tolerance of the experimental soybean plants. Previous studies with transgenic *Nicotiana tabacum* indicated that overproduction of Mn-SOD in mitochondria produced only a very small effect on O_3 tolerance (Van Camp et al. 1994). The present results suggest that the significance of a given antioxidant enzyme in O_3 tolerance primarily depends on the place of its action.

Several types of oxidative stresses (cold, paraquat, SO_2) have been found to involve an internal production of reactive oxygen species (Alscher et al. 1991). It has been suggested that a majority of O_3 decomposes into oxygen products in the apoplastic space and plasmalemma thereby resulting in negligibly small amounts of O_3 inside the cells (Laisk et al. 1989). Therefore, enhanced antioxidant capacities of external cell structures and cytosol are crucial for preventing O_3 damage in chloroplasts and mitochondria.

In the current study, APX had its greatest activity in the cytosol. Also, cAPX appeared to be more responsive to the O_3 treatments and had the highest activity in the O_3 tolerant cultivar. A positive correlation between cAPX activity and O_3 tolerance implies an importance of fast H_2O_2 removal in cytosol. However, the study did not evaluate a relative response of cytosolic SOD and GR activities and it is likely that without an adequate rate of AsA regeneration, the process of H_2O_2 scavenging will slow down. There were reports of increased O_3 sensitivity in *Nicotiana tabacum* with suppressed cAPX expression but no improvement in O_3 tolerance when cAPX was over-

expressed (Orvar and Ellis 1997). Chloroplastic APX may have limited effect on plant performance under elevated O_3. Torsethaugen et al. (1997) in their study with transgenic *Nicotiana tabacum* plants had shown that a high APX activity level in chloroplast does not guarantee enhanced O_3 tolerance. Under conditions of internal stress when chloroplast electron transport is affected, sAPX involvement in the stress tolerance appears to be reasonable. In the case of O_3 stress, H_2O_2 may appear first in the cytosol rather than in chloroplast, which would require enhanced cAPX activity. However, the method used in the current study did not allow us to detect differences between sAPX activities of the two soybean cultivars and draw any conclusion about a relationship between O_3 tolerance and sAPX activity.

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